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Molecular Characterization of the Melanocyte Lineage-specific Antigen gp100*

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The glycoproteins recognized by monoclonal antibody (mAb) NKI-beteb are among the best diagnostic markers for human melanoma because their expression is restricted to melanocytic cells. Recently, we isolated a cDNA clone, termed gp100-c1, which confers immunoreactivity not only to mAb NKI-beteb, but also to two other mAbs used to diagnose malignant melanoma, HMB-50 and HMB-45. In this report, we demonstrate that gp100-c1 cDNA encodes glycoproteins of 100 kDa (gp100) and 10 kDa (gp10) which are recognized by these mAbs in human melanoma cells. The translation product deduced from the open reading frame present in gp100-c1 cDNA is highly homologous to another melanocyte-specific protein, Pmel17. Nucleotide sequence analysis of genomic DNA indicates that the transcripts corresponding to gp100 and Pmel17 cDNAs originate from a single gene via alternative splicing. In all normal and malignant melanocytic cells analyzed, gp100 and Pmel17 RNAs are simultaneously expressed.

Melanoma is a neoplasm that originates from melanocytes, pigment-producing cells in the skin. Melanoma is a relatively immunogenic tumor, as demonstrated by the presence of both cytotoxic T lymphocytes (CTL)[1] (Knuth *et al.*, 1992) and antibodies (Mattes *et al.*, 1983) reacting with melanoma cells in patients. Characterization of the antigens recognized revealed that they include both tumor-specific antigens (van der Bruggen *et al.*, 1991) and the melanocyte differentiation antigens tyrosinase (Brichard *et al.*, 1993) and gp75 (Vijayasaradhi *et al.*, 1990). Both these differentiation antigens are localized in a distinct cellular organelle, the melanosome, and are involved in the synthesis of the pigment melanin (Hearing and Tsukamoto, 1991). To understand the potential role of immunological events in the pathogenesis and clinical course of melanoma, it is important to identify more of these antigens. This will not only result in the identification of potential targets for immune responses against melanoma, it may also lead to the identification of antigens involved in melanocyte differentiation and possibly transformation.

Many monoclonal antibodies have been raised against melanoma cells. Clinical and pathological analyses using these mAbs have led to the description of different steps in the transformation and progression of melanoma (reviewed by Ruiter *et al.* (1990)). Some of these mAbs define important markers in the initial diagnosis of melanoma, while others define melanoma progression antigens. Most of the antigens are expressed by both melanoma cells and melanocytes and are probably expressed during melanocyte differentiation. The melanocyte lineage-specific antigens recognized by mAb NKI-beteb are among the best diagnostic markers for human melanoma (Vennegoor *et al.*, 1988). NKI-beteb reacts with melanoma cells throughout tumor development and does not cross-react with other tumor cell types or normal cells, except for cells of the melanocytic lineage. The antigens recognized by NKI-beteb are glycoproteins of approximately 10 kDa (gp10) and 100 kDa (gp100). Ultrastructural analysis of NKI-beteb binding sites indicates that the antigens are localized in premelanosomal vesicles (Vennegoor *et al.*, 1988). Recently, we isolated a cDNA clone, gp100-c1, which confers immunoreactivity to NKI-beteb after expression in gp100-negative BLM melanoma cells (Adema *et al.*, 1993). Interestingly, two other mAbs used to diagnose malignant melanoma, HMB-50 (Vogel and Esclamado, 1988) and HMB-45 (Gown *et al.*, 1986), also react with BLM cells transfected with gp100-c1 cDNA (Adema *et al.*, 1993), indicating that all three mAbs recognize proteins encoded by a single cDNA. Herein, we report the molecular characterization of gp100-c1 cDNA and the melanocyte lineage-specific protein it encodes and discuss oncological and cell-biological aspects of the gp100 antigen.

MATERIALS AND METHODS

Cells and Monoclonal Antibodies—The melanoma cell lines Mel-2a, M14, MEWO, and BLM (Adema *et al.*, 1993) and the uveal melanoma cell line Mel 202 (Ksander *et al.*, 1991) have been described. Isolation of human melanocytes from breast or foreskin was performed by the method of Eisinger and Marko (1982) with modifications by (Smit *et al.*, 1993). NKI-beteb and HMB-50 have been described previously (Vennegoor *et al.*, 1988; Vogel and Esclamado, 1988). HMB-45 was purchased from Enzo Biochem.

DNA Constructs, Transfections, and Immunofluorescence—gp100-c1 cDNA was cloned in both orientations (pSVLgp100+ and pSVLgp100−) in the *Sma*I site of the eukaryotic expression vector pSVL (Pharmacia Biotech Inc.). pSVL contains the SV40 late promoter and polyadenylation site, as well as the SV40 origin of replication, allowing a very high copy number during transient expression in COS-7 cells. For the construction of the 3′ truncated gp100 transcription unit pSVLgp100+(ΔBS), we deleted the sequence between the *Bgl*II site in the 3′ part of gp100-c1 cDNA and the *Sac*I site in the vector. The resulting construct encodes a truncated gp100 protein in which the carboxyl-terminal 133 amino acids of gp100 are replaced by 4 amino acids (Arg-Ile-Gln-Thr) encoded by vector sequences. Transient expression of the constructs in COS-7 cells was performed by using 40 μg/ml Lipofectin reagent from Life Technologies, Inc. (Felgner *et al.*, 1987) and 7.5 μg of DNA. Transfected COS-7 cells were prepared for immunofluo-

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[1] The abbreviations used are: CTL, cytotoxic T lymphocyte; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; PCR, polymerase chain reaction; bp, base pair(s).

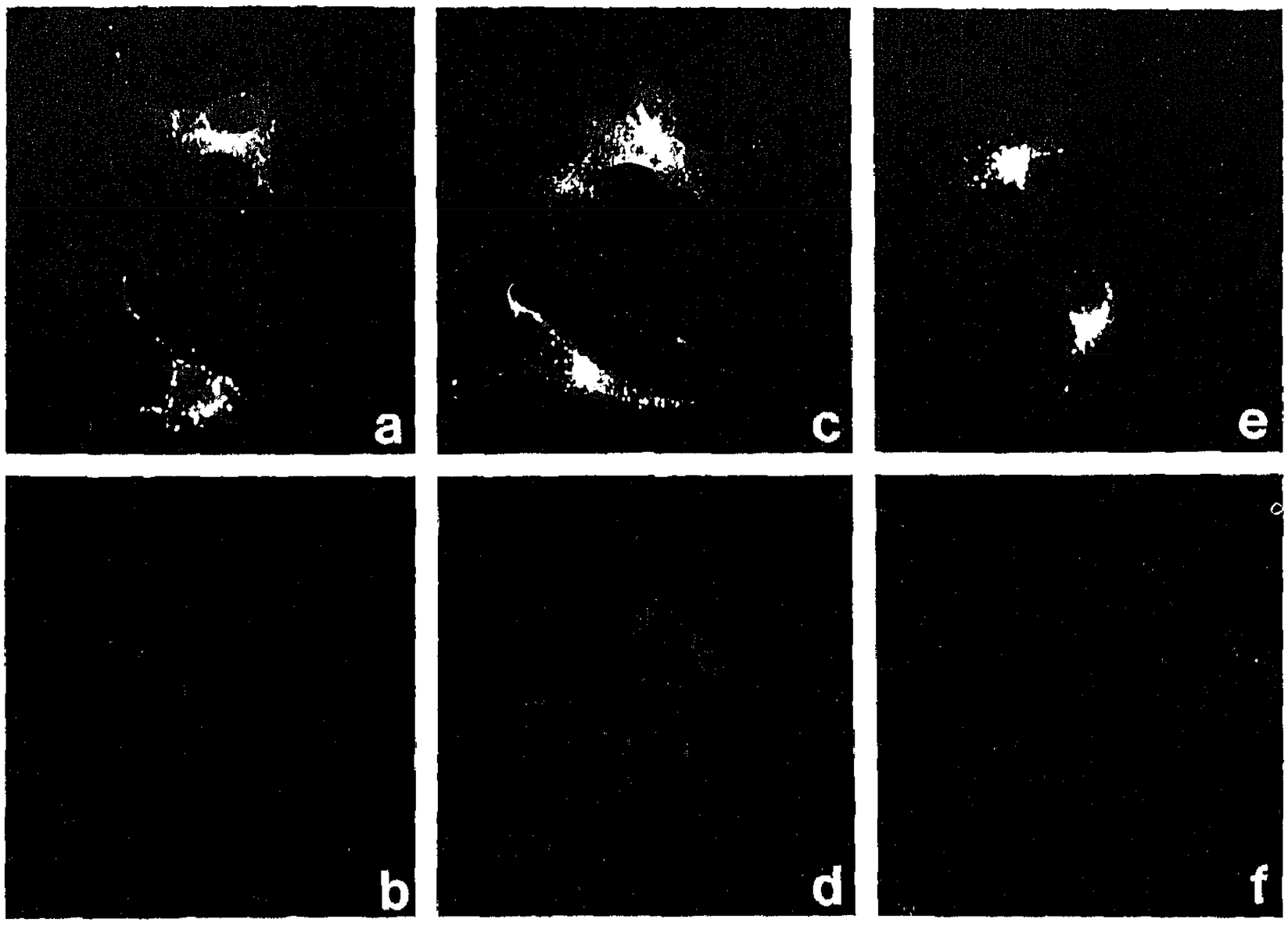

FIG. 1. **Expression of gp100-c1 cDNA in non-pigmented COS-7 cells.** COS-7 cells transfected with either gp100-c1 cDNA in the coding (pSVLgp100+; *a*, *c*, and *e*) or non-coding (pSVLgp100–; *b*, *d*, and *f*) orientation were analyzed for reactivity with NKI-beteb (*a* and *b*), HMB-45 (*c* and *d*), or HMB-50 (*e* and *f*), respectively. Indirect immunofluorescence (fluorescein isothiocyanate) was examined by confocal laser scanning microscopy. Magnification, × 40.

rescence as described previously (Adema *et al.*, 1993) and examined using a confocal laser scanning microscope at 488 nm (Bio-Rad MRC 600).

Metabolic Labeling, Immunoprecipitations, and V8 Protease Mapping—Immunoprecipitation experiments were performed on metabolically labeled (L-[^{35}S]methionine/cysteine; Amersham Corp.) cells as described by Vennegoor *et al.* (1988) using either NKI-beteb or HMB-50 covalently linked to protein A-Sepharose CL-4B beads (Pharmacia). In some experiments, tunicamycin (75 μg/ml, Calbiochem) was added during the prelabeling period and remained present during the metabolic labeling reaction (12.5 min). Immunoprecipitates were analyzed under reducing conditions by SDS-PAGE using 5–17.5% gradient gels. The relative molecular weight of the proteins was determined using co-electrophoresed, prestained markers (Life Technologies, Inc.). Gels were treated with 1 M sodium salicylate (pH 5.4) prior to autoradiography (Kodak XAR).

V8 protease mapping was performed using the procedure described by Cleveland *et al.* (1977). Briefly, gel slices containing the 100-kDa proteins were placed in the wells of a second SDS gel (10%) and overlaid with *Staphylococcus aureus* V8 protease (2.5 μg/sample, Miles Laboratories). After electrophoresis, gels were treated as described above.

Molecular Cloning of Part of the gp100/Pmel17 Gene—Part of the gp100/Pmel17 gene was amplified by PCR on human genomic DNA isolated from peripheral blood lymphocytes using the following primers: 1497/1516 (5′-TATTGAAAGTGCCGAGATCC-3′) and 1839/1857 (5′-TGCAAGGACCACAGCCATC-3′). The PCR products were subsequently amplified using a nested set of primers containing an additional *Eco*RI site (underlined) (5′-TATCTAGAATTCTGCACCAGATACTGAAG-3′ and 5′-TATCTAGAATTCTGCAAGATGCCCACGATCAG-3′) and cloned into the *Eco*RI site of pUC 18.

RNA Isolation and Analysis—Total RNA was isolated using the guanidine thiocyanate/cesium chloride procedure (Chirgwin *et al.*, 1979). cDNA was prepared using the GeneAmp RNA PCR kit (Perkin-Elmer), and PCR analysis was performed for 35 cycles in the presence of 3 mM $MgCl_2$ using primers 1497/1516 and 1839/1857 (see above) as described (Adema and Baas, 1991). The reaction products were analyzed by Southern blotting and hybridization to ^{32}P-labeled oligonucleotide probes as described previously (Adema and Baas, 1991). As probes, we used either a gp100-specific exon/exon junction oligonucleotide (5′-CTTCTTGACCAGGCATGATA-3′) or a Pmel17-specific oligonucleotide (5′-TGTGAGAAGAATCCCAGGCA-3′) that corresponds to 20 of the additional 21 nucleotides present in Pmel17 cDNA. In every hybridization experiment, a spot blot containing an oligonucleotide comprising the Pmel17 exon/exon junction (5′-GCTTATCATGC CTGTGCCTGGATTCTTCTCACAGGT-3′) was included as a control.

Nucleotide Sequence Analysis—gp100-c1 cDNA and genomic DNA clones were sequenced by the dideoxynucleotide sequencing method (Sanger *et al.*, 1977) using T7 DNA polymerase (Pharmacia). The sequence of both strands was determined in each case. Since the genomic DNA clones were obtained after PCR, the sequence of four independent clones was determined. Analysis of the DNA sequence was performed using the University of Winconsin Genetics Computing Group sequence analysis programs (Devereux *et al.*, 1984).

RESULTS

Expression of gp100-c1 cDNA in Non-pigmented COS-7 Cells Results in Immunoreactivity with mAbs NKI-beteb, HMB-50, and HMB-45—Expression of gp100-c1 cDNA in gp100-negative BLM melanoma cells results in immunoreactivity with the melanocyte lineage-specific mAbs NKI-beteb, HMB-50, and HMB-45 (Adema *et al.*, 1993). To determine whether expression of gp100-c1 cDNA in non-melanocytic cells also results in immunoreactivity with these mAbs, we performed transient expression experiments in COS-7 cells (monkey kidney fibroblasts) with constructs containing gp100-c1 cDNA in the coding or non-coding orientation. As shown in Fig. 1, only COS-7 cells transfected with the construct containing the cDNA in the coding orientation (COS-7/pSVLgp100+) reacted with all three mAbs. These data demonstrate that immunoreactivity with NKI-beteb, HMB-50, and HMB-45 after expression of gp100-c1 cDNA is not restricted to melanocytic cells. In addition, these data show that the COS expression system can be used for further biochemical characterization of the proteins encoded by gp100-c1 cDNA.

Analysis of the Proteins Encoded by gp100-c1 cDNA—To characterize the proteins encoded by gp100-c1 cDNA, COS-7/pSVLgp100+ cells were metabolically labeled and subjected to immunoprecipitation with NKI-beteb or HMB-50. As shown in Fig. 2, NKI-beteb (*panel A*) and HMB-50 (*panel B*) specifically detected proteins of approximately 100 kDa (95–110 kDa) in extracts of COS-7/pSVLgp100+ cells. The molecular mass of these proteins is similar (see also below) to those detected in extracts of metabolically labeled MEWO cells (Fig. 2), which express the antigens endogenously (Vennegoor *et al.*, 1988). Consistent with previous reports (Vennegoor *et al.*, 1988; Vogel and Esclamado, 1988), both mAbs also recognize a protein of 10 kDa in extracts of MEWO cells (Fig. 2, *lanes 6* and *7*). A protein of the same size reacted with NKI-beteb in COS-7/pSVLgp100+ cells (Fig. 2*A*, *lane 4*) and could be discerned with HMB-50 after prolonged exposure (not shown). No specific proteins were immunoprecipitated by either of the mAbs from extracts prepared

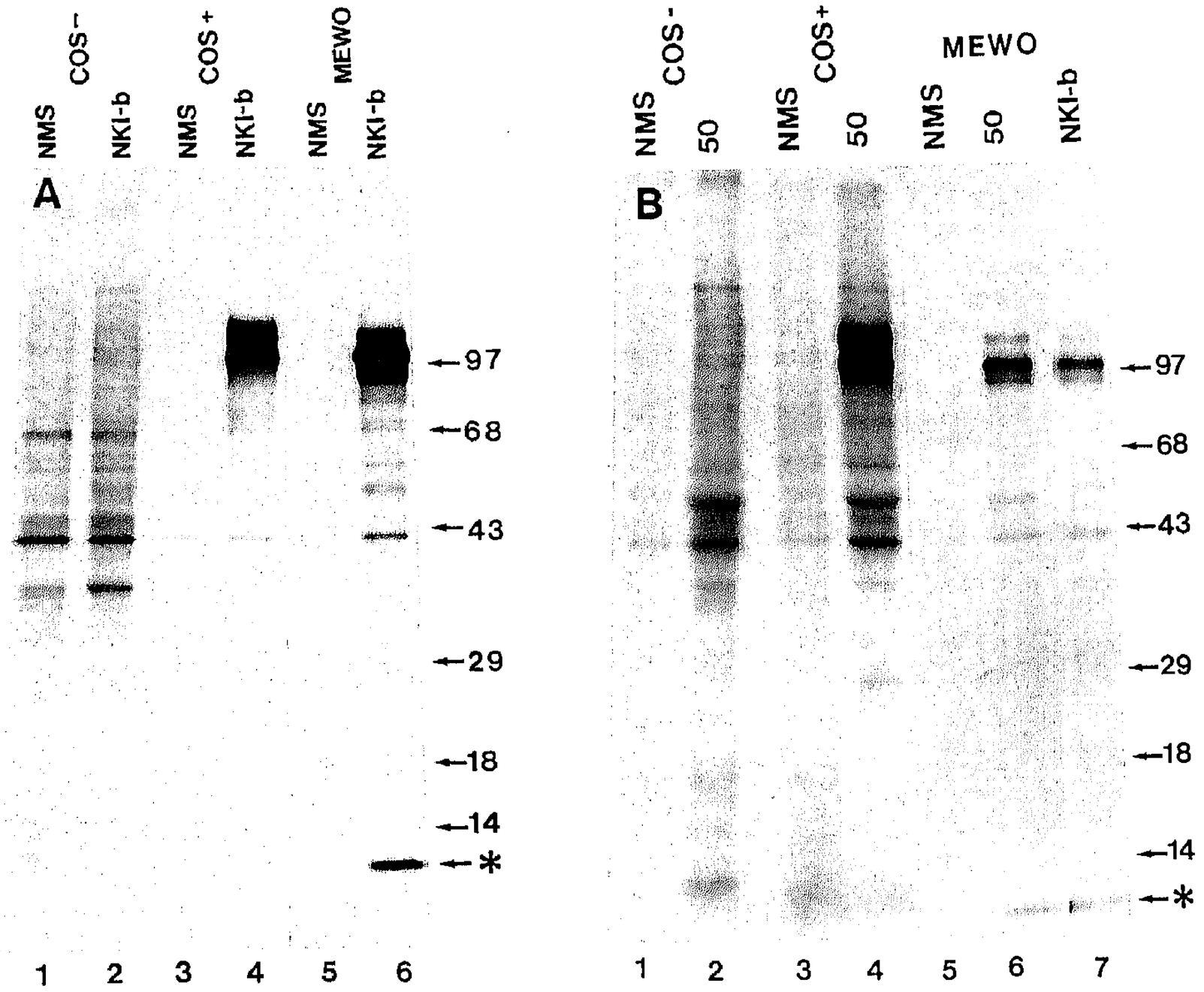

FIG. 2. **Proteins recognized by NKI-beteb (*panel A*) and HMB-50 (*panel B*) in extracts from gp100-c1 cDNA-transfected COS-7 cells.** MEWO cells (*MEWO*) and COS-7 cells transfected with either pSVLgp100+ (*COS+*) or pSVLgp100− (*COS−*) were metabolically labeled and subjected to immunoprecipitations using NKI-beteb (*NKI-b*), HMB-50 (*50*), or normal mouse serum (*NMS*) as indicated above each lane. Immunoprecipitated proteins were analyzed under reducing conditions by SDS-PAGE (linear gradient of 5–17% acrylamide) and visualized by autoradiography. The 10-kDa protein is indicated by an *asterisk*. The position and size (kDa) of prestained molecular weight markers are indicated.

from COS-7 cells transfected with the construct containing the cDNA in the non-coding orientation (Fig. 2). Comparison of the culture medium of metabolically labeled COS-7/pSVLgp100+ cells and MEWO cells revealed that both mAbs also recognized proteins of about 100 kDa (see also below) in the culture medium of these cells (Fig. 3). No proteins of 10 kDa were immunoprecipitated by the mAbs from the culture medium of COS-7/pSVLgp100+ cells, as has been shown for melanoma cells.

To exclude the possibility that the proteins detected by the mAbs are derived from endogenous genes induced after transfection with gp100-c1 cDNA, we performed immunoprecipitation experiments with COS-7 cells expressing a 3′ truncated gp100 transcription unit (see "Materials and Methods"). As shown in Fig. 4, proteins of approximately 85 kDa were detected by both mAbs in COS-7 cells expressing this construct, consistent with a deletion of 129 amino acids. This finding provides direct evidence that the 100-kDa protein recognized by NKI-beteb and HMB-50 in COS-7/pSVLgp100+ cells is encoded by gp100-c1 cDNA.

The 100-kDa Protein Encoded by gp100-c1 cDNA Is Identical to gp100—The proteins of about 100 kDa identified by NKI-beteb and HMB-50 in COS-7/pSVLgp100+ cells *versus* MEWO cells had a slightly different mobility when analyzed by SDS-PAGE (Fig. 2). This difference could be due to altered glycosylation, an event frequently observed in the COS expression system. Analysis of the proteins immunoprecipitated from MEWO cells and COS-7/pSVLgp100+ cells cultured in the presence of the glycosylation inhibitor tunicamycin demonstrated that in both cell types the size of the proteins of about 100 kDa was reduced to two protein bands of 90 and 85 kDa, confirming that the difference in mobility is due to altered glycosylation (not shown).

To provide further evidence that the proteins recognized by NKI-beteb in COS-7/pSVLgp100+ cells and MEWO cells are identical, we performed a V8 protease mapping experiment. As shown in Fig. 5, the same protein fragments were obtained after V8 protease digestion of the major 100-kDa protein isolated from COS-7/pSVLgp100+ cells or MEWO cells. We conclude from these data that gp100-c1 cDNA encodes the melanocyte lineage-specific glycoprotein gp100 recognized by NKI-beteb and HMB-50 in melanoma cells.

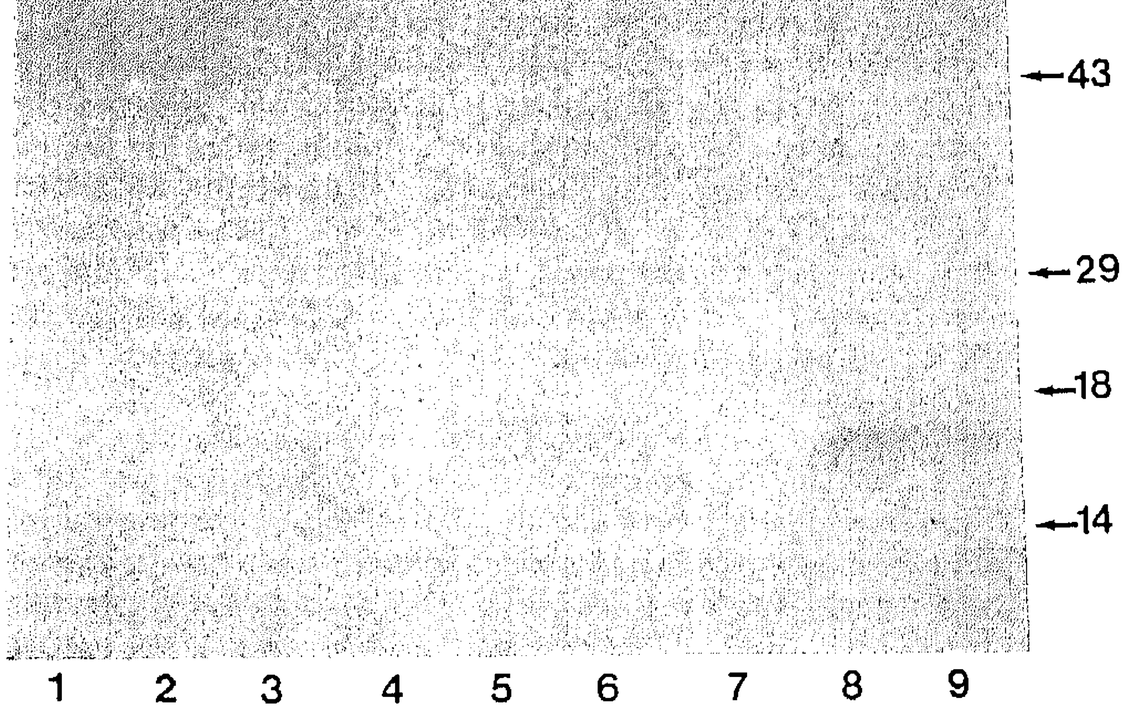

FIG. 3. **Proteins recognized by NKI-beteb and HMB-50 in culture medium from gp100-c1 cDNA-transfected COS-7 cells.** Culture medium from metabolically labeled MEWO cells (*MEWO*) or COS-7 cells transfected with pSVLgp100+ (*COS+*) or pSVLgp100− (*COS−*) was subjected to immunoprecipitations using NKI-beteb (*NKI-b*), HMB-50 (*50*), or normal mouse serum (*NMS*) as indicated above each lane. Analysis by SDS-PAGE and molecular weight markers were as described in Fig. 2.

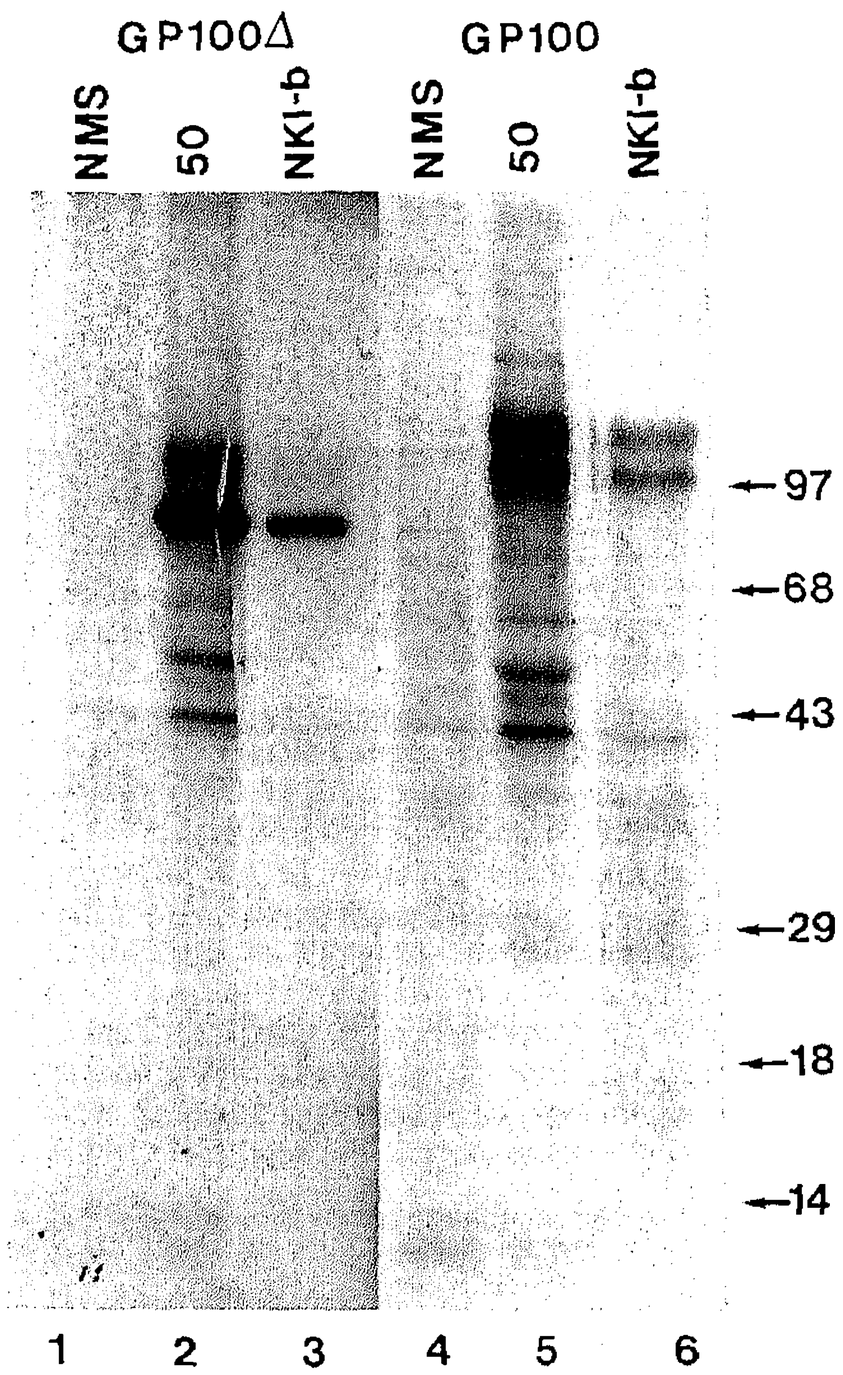

FIG. 4. **Proteins recognized by NKI-beteb and HMB-50 in COS-7 cells expressing a truncated gp100 transcription unit.** Extracts from metabolically labeled COS-7 cells transfected with pSVLgp100+ (*gp100*) or the 3′ truncated construct pSVLgp100+(ΔBS) (*gp100*Δ), see "Materials and Methods") were subjected to immunoprecipitations using NKI-beteb (*NKI-b*), HMB-50 (*50*), or normal mouse serum (*NMS*) as indicated above each lane. Analysis by SDS-PAGE and molecular weight markers were as described in Fig. 2.

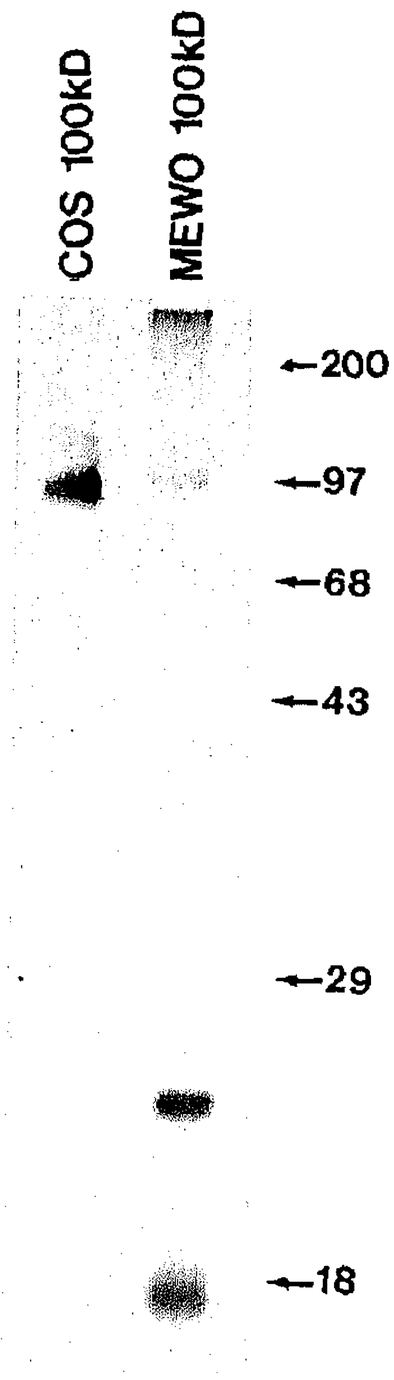

FIG. 5. **V8 protease mapping of the major 100-kDa protein detected by NKI-beteb in COS-7/pSVLgp100+ cells and MEWO cells.** Gel slices containing the major 100-kDa protein detected by NKI-beteb in MEWO cells (*lane 2*) and COS-7/pSVLgp100+ cells (*lane 1*) were subjected to V8 protease digestion as described by Cleveland *et al.* (1977). The resulting protein fragments were analyzed by SDS-PAGE (10%). Molecular weight markers were as indicated in Fig. 2.

gp100 Is a Type I Transmembrane Protein Highly Homologous to Pmel17—The nucleotide sequence of gp100-c1 cDNA was determined. It contains 2115 base pairs and terminates with a poly(A) tract of 15 nucleotides, preceded by the consensus polyadenylation sequence AATAAA (Proudfoot and Brownlee, 1976). An open reading frame extending from position 22 through 2007 is present in gp100-c1 cDNA. This open reading frame starts with an ATG codon within the appropriate sequence context for translation initiation (Kozak, 1987) and predicts a protein of 661 amino acids (Fig. 6). The amino-terminal 20 amino acids fit all criteria for signal sequences, including a potential cleavage site after Ala at position −1 (von Heyne, 1986), implying that mature gp100 contains 641 amino acids (approximately 70 kDa). Based on hydrophobicity plot analysis (Kyte and Doolittle, 1982), a single transmembrane domain bordered by charged residues is present in the carboxyl-terminal part (positions 571–591) of gp100. The predicted cytoplasmic domain is 45 amino acids long. Five putative *N*-linked glycosylation sites are present, consistent with gp100 being a glycoprotein. Furthermore, a histidine-rich domain (position 162–293), a threonine-rich domain (position 289–407) with repetitive amino acid sequences, and a cysteine-rich domain (position 455–546) are present.

A data base search (Pearson and Lipman, 1988; Altschul *et al.*, 1990) revealed that gp100 is almost identical to Pmel17, another melanocyte-specific protein (Kwon *et al.*, 1991). The amino acid differences between gp100 and Pmel17 consist of substitutions at position 254 (T → C/Pro → Leu) and 577 (C → G/Arg → Pro) and a stretch of 7 amino acids absent in gp100 at position 567 (see also Table II). A single nucleotide difference at position 762 (C → T) does not result in an amino acid substitution. gp100 is also 80% homologous to a putative protein deduced from a partial bovine cDNA clone (RPE-1) (Kim and Wistow, 1992) and 42% homologous to a chicken melanosomal matrix protein, MMP115 (Mochii *et al.*, 1991).

gp100 and Pmel17 Are Encoded by a Single Gene—The most striking difference between gp100 and Pmel17 cDNAs is the in-frame deletion of 21 bp in gp100 cDNA. Possibly, both cDNAs correspond to transcripts generated by alternative splicing of a single primary transcript. To test this hypothesis, we used PCR to analyze the genomic DNA corresponding to the part of the gp100 gene surrounding the putative alternative splice site. Comparison of the nucleotide sequence of this genomic DNA with the sequence of gp100-c1 cDNA revealed the presence of an intron (102 bp) just at the position of the 21-bp insertion in Pmel17 cDNA (Table I). The exon/intron boundaries nicely fit the consensus 5′ donor and 3′ acceptor splice site sequences (Padgett *et al.*, 1986). In the genomic DNA, the sequence comprising the additional 21 bp in Pmel17 cDNA is located directly upstream of this 3′ cleavage site and is preceded by an alternative 3′ acceptor splice site (Table I). Whereas the gp100-specific 3′ acceptor splice site fits the consensus sequence, the Pmel17-specific 3′ acceptor splice site appears to be suboptimal, in that it lacks a pyrimidine-rich region (Table I). Subop-

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   1  cgcggaatccggaagaacacaatggatctggtgctaaaaagatgccttcttcatttggctgtgataggtgctttgctggctgtgggggct
                        M  D  L  V  L  K  R  C  L  L  H  L  A  V  I  G  A  L  L  A  V  G  A
                                                                                   +1
  91  acaaaagtacccagaaaccaggactggcttggtgtctcaaggcaactcagaaccaaagcctggaacaggcagctgtatccagagtggaca
  24  T  K  V  P  R  N  Q  D  W  L  G  V  S  R  Q  L  R  T  K  A  W  N  R  Q  L  Y  P  E  W  T

 181  gaagcccagagacttgactgctggagaggtggtcaagtgtccctcaaggtcagtaatgatgggcctacactgattggtgcaaatgcctcc
  54  E  A  Q  R  L  D  C  W  R  G  G  Q  V  S  L  K  V  S  N  D  G  P  T  L  I  G  A  N  A  S
                                                                                      *
 271  ttctctattgccttgaacttccctggaagccaaaaggtattgccagatgggcaggttatctgggtcaacaataccatcatcaatgggagc
  84  F  S  I  A  L  N  F  P  G  S  Q  K  V  L  P  D  G  Q  V  I  W  V  N  N  T  I  I  N  G  S
                                                                  *                 *
 361  caggtgtggggaggacagccagtgtatccccaggaaactgacgatgcctgcatcttccctgatggtggaccttgcccatctggctcttgg
 114  Q  V  W  G  G  Q  P  V  Y  P  Q  E  T  D  D  A  C  I  F  P  D  G  G  P  C  P  S  G  S  W

 451  tctcagaagagaagctttgtttatgtctggaagacctggggccaatactggcaagttctagggggcccagtgtctgggctgagcattggg
 144  S  Q  K  R  S  F  V  Y  V  W  K  T  W  G  Q  Y  W  Q  V  L  G  G  P  V  S  G  L  S  I  G

 541  acaggcagggcaatgctgggcacacacaccatggaagtgactgtctaccatcgccggggatcccggagctatgtgcctcttgctcattcc
 174  T  G  R  A  M  L  G  T  H  T  M  E  V  T  V  Y  H  R  R  G  S  R  S  Y  V  P  L  A  H  S

 631  agctcagccttcaccattactgaccaggtgcctttctccgtgagcgtgtcccagttgcgggccttggatggagggaacaagcacttcctg
 204  S  S  A  F  T  I  T  D  Q  V  P  F  S  V  S  V  S  Q  L  R  A  L  D  G  G  N  K  H  F  L

 721  agaaatcagcctctgacctttgccctccagctccatgaccccagtggctatctggctgaagctgacctctcctacacctgggactttgga
 234  R  N  Q  P  L  T  F  A  L  Q  L  H  D  P  S  G  Y  L  A  E  A  D  L  S  Y  T  W  D  F  G

 811  gacagtagtggaaccctgatctctcgggcacttgtggtcactcatacttacctggagcctggcccagtcactgcccaggtggtcctgcag
 264  D  S  S  G  T  L  I  S  R  A  L  V  V  T  H  T  Y  L  E  P  G  P  V  T  A  Q  V  V  L  Q

 901  gctgccattcctctcacctcctgtggctcctccccagttccaggcaccacagatgggcacaggccaactgcagaggcccctaacaccaca
 294  A  A  I  P  L  T  S  C  G  S  S  P  V  P  G  T  T  D  G  H  R  P  T  A  E  A  P  N  T  T
                                                                                      *
 991  gctggccaagtgcctactacagaagttgtgggtactacacctggtcaggcgccaactgcagagccctctggaaccacatctgtgcaggtg
 324  A  G  Q  V  P  T  T  E  V  V  G  T  T  P  G  Q  A  P  T  A  E  P  S  G  T  T  S  V  Q  V

1081  ccaaccactgaagtcataagcactgcacctgtgcagatgccaactgcagagagcacaggtatgacacctgagaaggtgccagtttcagag
 354  P  T  T  E  V  I  S  T  A  P  V  Q  M  P  T  A  E  S  T  G  M  T  P  E  K  V  P  V  S  E

1171  gtcatgggtaccacactggcagagatgtcaactccagaggctacaggtatgacacctgcagaggtatcaattgtggtgctttctggaacc
 384  V  M  G  T  T  L  A  E  M  S  T  P  E  A  T  G  M  T  P  A  E  V  S  I  V  V  L  S  G  T

1261  acagctgcacaggtaacaactacagagtgggtggagaccacagctagagagctacctatccctgagcctgaaggtccagatgccagctca
 414  T  A  A  Q  V  T  T  T  E  W  V  E  T  T  A  R  E  L  P  I  P  E  P  E  G  P  D  A  S  S

1351  atcatgtctacggaaagtattacaggttccctgggccccctgctggatggtacagccaccttaaggctggtgaagagacaagtcccccctg
 444  I  M  S  T  E  S  I  T  G  S  L  G  P  L  L  D  G  T  A  T  L  R  L  V  K  R  Q  V  P  L

1441  gattgtgttctgtatcgatatggttccttttccgtcaccctggacattgtccagggtattgaaagtgccgagatcctgcaggctgtgccg
 474  D  C  V  L  Y  R  Y  G  S  F  S  V  T  L  D  I  V  Q  G  I  E  S  A  E  I  L  Q  A  V  P

1531  tccggtgaggggggatgcatttgagctgactgtgtcctgccaaggcgggctgcccaaggaagcctgcatggagatctcatcgccagggtgc
 504  S  G  E  G  D  A  F  E  L  T  V  S  C  Q  G  G  L  P  K  E  A  C  M  E  I  S  S  P  G  C

1621  cagccccctgcccagcggctgtgccagcctgtgctacccagcccagcctgccagctggttctgcaccagatactgaagggtggctcgggg
 534  Q  P  P  A  Q  R  L  C  Q  P  V  L  P  S  P  A  C  Q  L  V  L  H  Q  I  L  K  G  G  S  G

1711  acatactgcctcaatgtgtctctggctgataccaacagcctggcagtggtcagcacccagcttatcatgcctggtcaagaagcaggcctt
 564  T  Y  C  L  N  V  S  L  A  D  T  N  S  L  A  V  V  S  T  Q  L  I  M  P  G  Q  E  A  G  L
                        *                                                             ___  ___
1801  gggcaggttccgctgatcgtgggcatcttgctggtgttgatggctgtggtccttgcatctctgatatataggcgcagacttatgaagcaa
 594  G  Q  V  P  L  I  V  G  I  L  L  V  L  M  A  V  V  L  A  S  L  I  Y  R  R  R  L  M  K  Q
      ___  ___  ___  ___  ___  ___  ___  ___  ___  ___  ___  ___  ___  ___  ___  ___  ___  ___

1891  gacttctccgtaccccagttgccacatagcagcagtcactggctgcgtctaccccgcatcttctgctcttgtcccattggtgagaatagc
 624  D  F  S  V  P  Q  L  P  H  S  S  S  H  W  L  R  L  P  R  I  F  C  S  C  P  I  G  E  N  S

1981  cccctcctcagtgggcagcaggtctgagtactctcatatgatgctgtgattttcctggagttgacagaaacacctatatttcccccagtc
 654  P  L  L  S  G  Q  Q  V

2071  ttccctgggagactactattaactgaaataaatactcagagcctgaaaaaaaaaaaaaaa
```

FIG. 6. **Nucleotide sequence of gp100-c1 cDNA and the deduced amino acid sequence.** The poly(A) addition site is indicated in *italics*. The deduced amino acid sequence contains a putative signal peptide (*underlined*). The first amino acid of mature gp100 is designated +1. Putative *N*-linked glycosylation sites are indicated by an *asterisk*, whereas the transmembrane region is *underlined*.

TABLE I

Genomic organization of part of the human gp100/Pmel17 gene

A and A′ represent the introns that are removed in transcripts corresponding to gp100-c1 cDNA and Pmel17 cDNA, respectively. Exon sequences are indicated in uppercase and intron sequences as lowercase letters. The best fit to the branch point sequence (Ruskin *et al.*, 1984) is underlined.

Intron	Size	
	nt	
A	102	CATGCCTGgtaggtcc.....agacactgagtgaagcagtgcctgggattcttctcacagGTCAAAG
A′	81	CATGCCTGgtaggtcc.....gggcagctggcaagcagcagacactgagtgaagcagTGCCTGG

timal RNA processing sites are present in many alternatively processed messenger RNA precursors and have been implicated to function in regulation of alternative RNA processing (reviewed by Green (1991)). These data prove that gp100 and Pmel17 transcripts are generated by alternative splicing of a single primary transcript and thus originate from a single gene.

Expression of gp100 and Pmel17 RNAs in Cells of the Melanocytic Lineage—The finding that gp100 and Pmel17 RNAs arise by alternative splicing of a single primary transcript raises the question of whether this occurs in a regulated manner. Previously, we showed that an RNA species of 2.5 kilobases is the major RNA product detected by gp100-c1 cDNA on North-

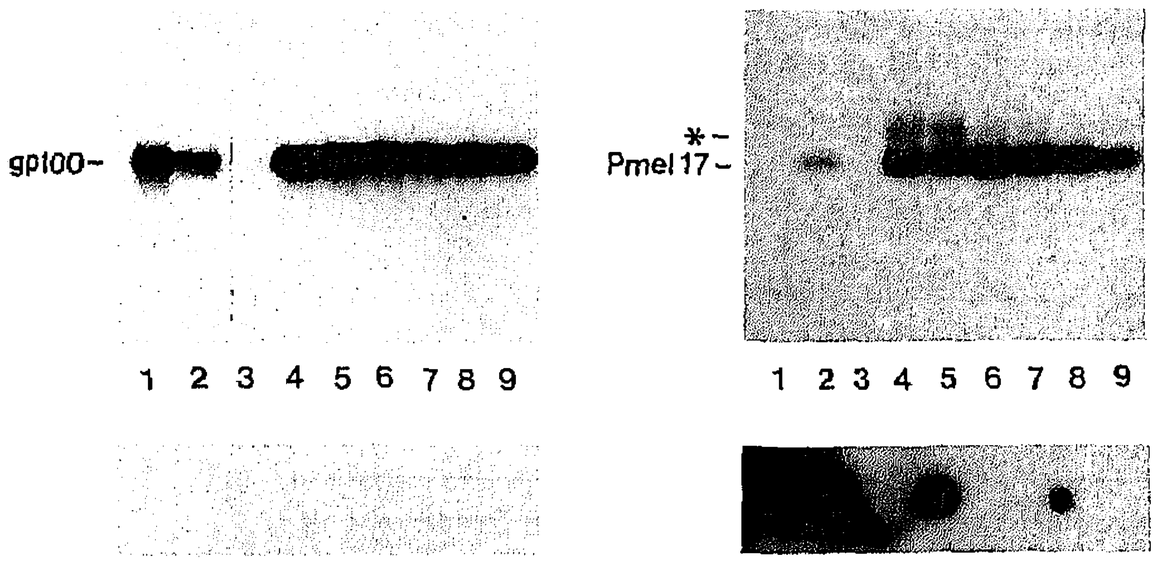

FIG. 7. **Expression of gp100 and Pmel17 RNA in cells of the melanocytic lineage.** Reverse transcriptase/PCR was performed on RNA isolated from MEWO (*lanes 2*), BLM (*lanes 3*), M14 (*lanes 4*), Mel2a (*lanes 5*) cutaneous melanoma cells, the uveal melanoma cells Mel 202 (*lanes 9*), neonatal melanocytes (*lanes 6* and *7*), and adult (*lanes 8*) melanocytes. As a control, PCR was performed on gp100-c1 cDNA (*lanes 1*). The reaction products were analyzed by Southern blotting and hybridization to either a gp100-specific (*left panel*) or a Pmel17-specific (*right panel*) oligonucleotide probe. As a control, both probes were hybridized to a spot blot containing different amounts (100, 10, and 1 ng) of the Pmel17-specific exon/exon junction. The position of the DNA species corresponding to Pmel17 and gp100 spliced products as well as unspliced material and/or contaminating genomic DNA (*) are indicated. Note that the gp100-specific exon/exon junction probe does not react with DNA species corresponding to unspliced material/genomic DNA.

ern blots containing RNA isolated from melanocytic cells. Essentially the same results were obtained by Kwon *et al.* (1987) using Pmel17–1 cDNA as a probe. However, neither of the probes discriminate between gp100 and Pmel17 RNAs. Therefore, we performed a reverse transcriptase/polymerase chain reaction assay followed by Southern blotting and hybridization to either a gp100-specific exon/exon junction or a Pmel17-specific oligonucleotide probe (see "Materials and Methods"). As shown in Fig. 7, gp100 and Pmel17 spliced products were both detected in three out of four cutaneous melanoma cells (*lanes 2, 4, and 5*) and in uveal melanoma cells (*lanes 9*), as well as in neonatal (*lanes 6* and *7*) and adult melanocytes (*lanes 8*). Both spliced products were also present in three different primary melanomas (not shown). No products were detected with either probe in gp100-negative BLM melanoma cells (*lanes 3*). These results demonstrate that in all melanocytic cells examined, gp100 and Pmel17 RNAs are expressed simultaneously.

DISCUSSION

Herein, we demonstrate that gp100-c1 cDNA encodes the melanocyte lineage-specific antigens recognized by mAbs NKI-beteb, HMB-50, and HMB-45, which are valuable diagnostic markers for melanoma. Expression of gp100-c1 cDNA in COS-7 cells demonstrates that NKI-beteb and HMB-50 recognize the same proteins of 100 and 10 kDa. Comparison of the 100-kDa protein in these cells and MEWO melanoma cells by V8 protease digestion demonstrates that they have the same protein backbone. The relationship between the 100- and 10-kDa proteins is not clear at present. The 10-kDa protein may be derived from the 100-kDa protein either by specific proteolytic processing or by degradation. Two findings lend support to the latter explanation. (*a*) The amount of 10-kDa proteins relative to the amount of 100-kDa proteins varies considerably between experiments, and (*b*) the 10-kDa protein can only be detected after a labeling period of 2 h while the 100-kDa protein is already present in the culture medium after 1 h (Esclamado *et al.*, 1986; results not shown).

In addition to NKI-beteb and HMB-50, HMB-45 also reacts with gp100-transfected COS-7 cells. HMB-45 has been reported to immunoprecipitate proteins of 10 kDa from extracts and of 100 kDa from culture medium of melanoma cells, both of which comigrate with those detected by HMB-50 and NKI-beteb (Esclamado *et al.*, 1986; Vennegoor *et al.*, 1988). In addition, an additive enzyme immunoassay revealed an additivity index of 91% for the mixture of HMB-45 and NKI-beteb (Vennegoor *et al.*, 1988). For our study, HMB-45 could not be obtained in sufficient amounts to directly analyze the proteins it detects in COS-7/pSVLgp100+ cells. However, the combined data of the authors mentioned above and those described herein indicate that the antigens recognized by HMB-45 are also encoded by gp100-c1 cDNA.

Proteins Homologous to gp100—A data base search revealed that gp100 is almost identical with the melanocyte-specific protein Pmel17. The cDNA encoding Pmel17 was isolated from a λ gt11 melanocyte cDNA library (Kwon *et al.*, 1987; 1991). The most striking difference between gp100-c1 and Pmel17 cDNA consists of an in-frame deletion of 21 bp in gp100-c1 cDNA. Nucleotide sequence analysis of part of the gene encoding gp100 demonstrates that both cDNAs correspond to transcripts originating from a single gene via alternative splicing. A single 5′ donor splice site is used in combination with two different, partially overlapping, 3′ acceptor splice sites. No regulated expression of gp100 and Pmel17 mRNAs in cells of the melanocytic lineage has been observed; cells either expressed neither of the mRNAs or both. These data are consistent with previous results obtained by Kwon *et al.* (1987, 1991), indicating that the gene encoding Pmel17, and hence the gp100 gene, is a single-copy gene that maps to human chromosome 12 (region 12 pter-q21). Three other nucleotide differences have been detected between gp100 and Pmel17, two of which give rise to an amino acid substitution. They may represent allelic variations or polymorphisms, but we cannot exclude the possibility that they result from mutations.

In addition to Pmel17, gp100 was found to be 80% homologous to the putative protein (RPE1) product encoded by a partial bovine cDNA isolated from retinal pigment epithelium (RPE) (Kim and Wistow, 1992). Bovine RPE has been shown to react with HMB-50 (Kim and Wistow, 1992) and human RPE with HMB-45 (Kapur *et al.*, 1992). Since the RPE1 protein is 80% homologous to gp100 and lacks the 7 amino acids present in Pmel17 (Table II), it may well represent the bovine homologue of gp100.

Another data base entry showing significant homology (42%) to gp100 is the melanosomal matrix glycoprotein MMP115 isolated from chicken RPE (Mochii *et al.*, 1991). MMP115 localizes, as gp100, in melanosomal vesicles. No function has been reported for MMP115. The amino-terminal sequence (670 amino acids) of MMP115 is 46% homologous to the corresponding part in gp100. The carboxyl-terminal part of MMP115 (84 amino acids) is only 13% homologous to gp100 and does not contain a transmembrane domain. Strikingly, the homology between gp100 and MMP115 decreases just at the site of the 7-amino acid insertion in Pmel17 (Table II). Perhaps MMP115 represents a soluble form of the chicken homologue of gp100 still to be discovered in man.

A data base search with the gp100 amino acid sequence or parts of this sequence did not reveal the presence of any known functional domain. The spacing of the cysteines in the cysteine-rich region that determines its tertiary fold is highly conserved between gp100/Pmel17, RPE1, and MMP115 (Table II) but is distinct from the ones found in other protein families, *e.g.* the integrin (Kishimoto *et al.*, 1989) or nerve growth factor receptor families (Mallett *et al.*, 1991). Therefore, the cysteine-rich domain present in the Pmel17/gp100 family may represent a novel structural or functional (interaction with other proteins) domain.

TABLE II

Alignment of the carboxyl-terminal part of members of the gp100/Pmel17 family

Identical amino acids (—) and gaps (*) are indicated. Conserved cysteine residues (#) are indicated as well.

```
                    #                                                    #
Gp100               PLDCVLYRYGSFSVTLDIVQGIESAEILQAVPS***GEGDAFELTVSCQGGLPKEA
Pmel17              ------------------------------***--------------------
RPE1                ------------L-----*S----------S-***S------------------
MMP115              -TG-------T--TE-N-------VA-V-V--AAPE-S-NSV----T-E-S--E-V

                    #       #       #       #                  #
Gp100               CMEISSPGCQPPAQRLCQPVLPSPACQLVLHQILKGGSGTYCLNVSLADTNSLAVV
Pmel17              ------------------------------------------------------
RPE1                --D-------L---------P-----------V----------------A----M-
MMp115              -TVVADAE-RTAQMQT-SA-A-A-G-----R-DFNQ*--L--------NG-G---A

Gp100               STQLIMP*******GQEAGLGQVPLIVGILLVLMAVVLASLIYRRRLMKQ**DFSV
Pmel17              -------VPGILLT---------R-------------------------**----
RPE1                ----V--*******------R-A--F-------T-LL------------GSEVPL
MMP115              --HVAVGSIPSRQWHHAHRGAALGTAH-RCSGHRCLH-PPCEVQPAAAHSPHGPPA

Gp100               PQLPHSSSHWLRLPRI*FCSCPIGENSPLLSGQQV
Pmel17              ----------------*------------------
RPE1                -----GRTQ-----WV*-R------SK--------
MMP115              ---AAPRCYPAFAAAPG-WGGSQWRKQ-PARANA-
```

Melanocyte-specific Antigen gp100: Cell-biological and Oncological Significance—The synthesis of melanin in melanocytes is a multistep process regulated at different levels (reviewed by Hearing and Tsukamoto (1991)). Tyrosinase (EC 1.14.10.1) is the key enzyme in this pathway and catalyzes the initial steps in the cascade of reactions leading to the production of melanin from the amino acid tyrosine. Two other members of the tyrosinase family are TRP-1 or gp75 (Shibahara *et al.*, 1986; Vijayasaradhi *et al.*, 1990) and TRP-2 (Jackson *et al.*, 1992), the latter of which contains 3,4-dihydroxy phenylalanine(DOPA)chrome tautomerase (EC 5.3.2.3) activity (Tsukamoto *et al.*, 1992). The combined data on gp100 and Pmel17 imply that they are also related to this process; 1) both proteins are only expressed in pigmented cells (Kwon *et al.*, 1987; Vennegoor *et al.*, 1988), 2) gp100 is present in melanosomal vesicles (Vennegoor *et al.*, 1988), and 3) an increase in the amount of transcripts derived from the gp100/Pmel17 gene correlates with increasing levels of melanization (Kwon *et al.*, 1987). Preliminary data indicate that Pmel17 also reacts with the anti-gp100 mAbs and is localized in melanosomes. Cumulatively, these data support a role of both gp100 and Pmel17 in melanization, either as enzymes regulating the quality or quantity of melanin synthesis or as structural components of the melanosome. The possibility that these proteins play a role in malignant transformation is not likely, but it cannot be excluded.

Melanoma is a relatively immunogenic tumor, demonstrated by the presence of both CTL (reviewed by Knuth *et al.*, 1992) and antibodies (Mattes *et al.*, 1983) against melanoma cells in patients. The recent finding that some CTL clones react not only with melanoma cells but also with normal melanocytes suggests that tissue-related antigens can also be recognized (Anichini *et al.*, 1993). Moreover, Brichard *et al.* (1993) showed that a peptide derived from tyrosinase is recognized by a CTL clone. Recently, we have identified gp100-specific tumor-infiltrating lymphocytes in a melanoma patient (Bakker *et al.*, 1994). These data demonstrate that besides its value as a tumor marker, gp100 may also serve as a target for specific immunotherapy against melanoma, provided that no unacceptable side effects are observed against normal tissues harboring pigmented cells.

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